TRANSLATION NO. 597

DATE: July 1968

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

DETECTION OF BOTULINUM TOXIN, ARTIFICALLY INTRODUCED INTO FOOD PRODUCTS BY MEANS OF THE PHAGOCYTIC INDEX DETERMINATION

By V.R. Savin

When studying the mechanism of the sensitizing action of botulinum toxin, Minervin, Shak and Chervyakova established that during test-tube experiments the toxin sharply inhibits the phagocytic activity of rabbit leucocytes. According to these authors, this property of the toxin is of a strictly specific character, as it becomes neutralized by the specific anti-botulinum serum of a corresponding type. Naturally, it seemed to be of interest to investigate to what degree these observations could be used in the practical control of botulism.

In his instructions on the use of the phagocytic index determination for the detection of the botulinum toxin, Minervin states that this method may be applied in the case of food products. The purpose of the present research was the study of the botulinum toxin, and the detection and establishment of its type through the phagocytic index determination in food products, such as sausage, ham, herring and canned food. For a satisfactory solution of this problem, a great number of objects must be investigated and the food products must contain exact quantities of toxin. In this paper we present observation on the detection of toxin which was artificially introduced in definite quantities into the food products investigated. Simultaneously, we studied the relative sensitivity of the method of phagocytic index determination and of the biological test widely used in the detection of the botulinum toxin.

We tested the phagocytic activity of botulinum toxin of type A (strain No. 98) and type B (strain no. 255).

The tests were set up as follows:

19 parts of the physiological solution and 1 part of the toxin (of either of the two types) were added to 5 gr. of the food product. The mixture was ground, and allowed to settle over a period of two hours at room temperature. After this the liquid portion was decanted into centrifuge test-tubes by means of sterile Pasteur pipettes. The contents of the test-tubes were centrifuged, and the supernatant liquid decanted and diluted as required.

In the first series of tests we studied the influence on the phagocytic activity of the rabbit leukocytes of the extracts, prepared from the canned fish, meat and vegetables to which type A toxin was added in dilutions of 1:25000, 1:50000, 1:75000 and 1:100000. The MLD of the toxin according to the date of a biological test was 1:60000 for mice. Each test was accompanied by a check in respect to the neutralization through the anti-botulinum serum of type A and B as well as normal equine serum. The mixtures containing serum were kept in a thermostat for 20 minutes at 37°, after which the phagocytosis reaction was carried out. Addition of the botulinum

... 2

toxin type A inactivated in a water bath at 100° for 30 minutes, as well as the usual phagocytosis reaction, in which physiological saline was substituted for the toxin, and these tests.

A total of 28 experiments was performed (312 investigations), 17 of which were carried out with canned fish, 5 with canned vegetables and 6 with canned meat.

In all cases, independently of the nature of the canned product investigated, we observed that the botulinum toxin sharply inhibited phagocytosis and, that this action of the toxin was quite specific in character. This manifested itself in two ways; first, the phagocytic activity of leucocytes increased with dilution with physiological solution, and after inactivation in a water bath for 30 minutes at 100°, the toxin lost its inhibiting action on the phagocytosis. The specific nature of the effect of botulinum toxin on phagocytosis was also confirmed by the fact that the phagocytic activity of rabbit leucocytes returned to normal, following an introduction of appropriate anti-botulinum serum into the reaction. It is necessary to note that the method of a biological test is considerably inferior to the method of the phagocytic index determination. The results of the tests which clearly demonstrate this are presented in table 1.

In the second series of experiments we studied the influence on the phagocytic activity of rabbit leucocytes of extracts, prepared from canned food products, to which botulinum toxin type B (strain no. 255) had been added. It should be pointed out that this strain produced a toxin of a very weak titre (1/60 milliliter for white mice). In this series of tests, we mixed citrated rabbit blood with extract of the canned product, as well as with the extract containing toxin in the following dilutions: 1:25, 1:50, 1:75, 1:100, 1:150 and 1:250. As in the preceding tests, the extracts containing toxin in a dilution of 1:25 were kept in a thermostat for 30 minutes together with the antibotulinum serums of type A and B and the normal equine serum.

The test was accompanied by controls consisting of introduction into white mice 1) extract of the canned product, 2) the same extract mixed with toxin in dilutions indicated above, and 3) extracts containing toxin mixed with anti-botulinum serums A,B, and normal equine serum. Inactivated extracts and the extracts containing the toxin were also used as controls. The total number of tests set up with toxin of type B was 24, in three of which we used canned meat, in 6 canned vegetables and in 15 canned fish. Following an addition of the extracts containing the botulinum toxin of type B. we observed, irrespective of the type of canned product used, a sharp drop in the phagocytic activity of the leucocytes in the rabbit blood, in all cases.

Extracts containing toxin B, added in a dilution of 1:25, and kept preliminarily in a thermostat for 30 minutes at 37° with the anti-botulinum serum of type A and a normal equine serum, also showed an inhibiting effect on phagocytosis. The type-specific anti-serum added to the extract containing the toxin of type B, induced a sharp intensification of the phagocytic activity of leucocytes in the rabbit blood. The extracts prepared from the canned food, but containing no botulinum toxin, as well as the extracts to which a toxin inactivated through heating was added, had no noticeable effect on phagocytosis.

... 3

Biological tests performed simultaneously with these experiments proved in all cases to fall behind the method of the phagocytic index determination. Since the results of these experiments are more or less identical with those presented in table 1 for the toxin of type A, we do not present a special table. Comparing the results of the determination of the toxin B (using the method of the phagocytic index) with the data of the biological test, it can be stated that the first method proved to be much more sensitive, especially if one considers the fact that the extracts, containing the toxin in dilutions 1:25, 1:50, 1:75, 1:100, 1:150, 1:200 were further diluted by a factor of 5 by the ingredients taking part in the reaction of phagocytosis. During the biological test the mice usually died following subcutaneous injection of an extract containing toxin in a dilution of 1:25 (for the canned meat), 1:25 and 1:50 (for the canned vegetables) and occasionally 1:75 (for the canned fish), whereas with the aid of the phagocytic index we succeeded in detecting the presence of toxin in dilutions of 1:100 1:150 and 1:200 (depending on the type of the canned goods). It can be concluded that the method of the phagocytic index is about 10 to 20 times more sensitive than the biological test.

Taking into account the fact that the reaction of phagocytosis with the canned goods may be affected by non-specific factors, such as products of the vital activity of microorganisms contained in the canned food, p^H of the medium, salts, etc. we set up the test with the normal preserved food; at the beginning of the test we added botulinum toxin A or B to the food in question, similarly to the previously described test with canned goods. In these tests the reaction of phagocytosis was set up not only with the extracts of canned meat, but also with sausage, ham and herring. We conducted a total of 30 such experiments (427 investigations of phagocytosis).

In the first series of experiments, extracts prepared from the contents of normal cans to which the botulinum toxin of type A had been added, were subjected to an investigation in respect to their ability to affect the phagocytic activity of the leucocytes. These tests, similarly to those described earlier, showed that the reaction of phagocytosis was most clearly and intensely inhibited following addition of toxin in the following dilutions: 1:25000, 1:50000, 1:75000, 1:100000. The phagocytic index decreased 2 to 12 times, depending on the type of canned food investigated. The sharpest drop in the phagocytic activity of leucocytes was observed when extracts prepared from canned vegetables were added to the blood. The inhibitory action on phagocytosis was also noticed when an extract, infected with the toxin A, was added to the blood. Prior to the experiment this extract was kept for 20 minutes with the anti-serum B or with normal equine serum in a thermostat at 37°. When an extract, which had been kept in a thermostat with the type-specific serum A was added to the blood sharp intensification of phagocytosis occurred. When extracts containing the toxin which had been subjected to inactivation at 100° for 20 minutes were added, phagocytosis was similar to the control reaction.

The biological test on white mice, conducted in two experiments parallel to the experiments with the toxin A, indicated considerably lower sensivity of this test in comparison with the phagocytosis test. Thus, during the biological test the animals died following an introduction of toxin in a

... 4

concentration of 1:25000, whereas the phagocytic index indicated the presence of the toxin, even in a dilution of 1:100000. Furthermore, taking into account the fact that the toxin used in the test had been diluted 5 times by the ingredients that participated in the reaction of phagocytosis, we must conclude that the sensivity of leucocytes of the rabbit blood to the toxin is about 20 times higher than that of the biological test.

In the next series of experiments, using similar methods, we studied the effect of the extracts of food products to which the toxin of type B was added on the phagocytic activity of rabbit leucocytes.

In those experiments as in the previous ones, we observed a sharp inhibition of phagocytosis, as compared with the control, in the mixtures to which an extract containing the toxin in concentrations of 1:25, 1:50, 1:75, 1:100 and 1:150 was added. With progressing dilution of the toxin an intensified phagocytic activity of leucocytes was observed, which, for the extract, containing the toxin in a dilution of 1:200 approached that of the control. When adding the B-type specific serum, the phagocytic index was twice or 2½ times higher than in the case, when serum A and normal equine serum were used. The results of these tests are shown in table 2.

The biological test conducted simultaneously in two cases on white mice, indicated a considerably lesser sensivity to the toxin of type B, as compared with the method of determination of the same toxin with the aid of the phagocytic index. In this case too, if the dilution of the toxin by ingredients participating in the test of determination of the phagocytic index are taken into account, the biological test proved to be 10 to 20 times less sensitive than the method of the phagocytic index. Summing up the presented data, we may note that the extracts from canned food and other products containing no toxin did not affect the phagocytic function of the leucocytes, whereas the same extracts induced a sharp inhibition of phagocytosis after the addition of the toxin. In the control tests with extracts containing toxin but subjected to thermal inactivation, the indices of phagocytosis were approximately the same as in the physiological solution.

CONCLUSIONS

1. Phagocytic index may be used for the detection of the botulinum toxin of types A and B in the food products, along with the biological test.

2. Botulinum toxins may be detected with the aid of the phagocytic index during an investigation of various canned goods.

3. With the aid of the reaction of phagocytosis considerably smaller quantities of the botulinum toxins may be revealed than with the aid of a biological test; therefore, the latter must be considered less sensitive as compared with the method of the phagocytic index determination.

... 5

4. The determination of the presence of a botulinum toxin in canned foods with the aid of the phagocytic index requires considerably less time (2-3 hours) as compared with the biological test.

5. With the aid of the method of the phagocytic index determination it is not only possible to detect the presence of the botulinum toxin but also to determine its type, which is quite important for therapeutic purposes.

TABLE 1.

Phagocytic index

Name of the product	Physiological solution	Native extract	Inactivated extract	Extract plus toxin 1:25000 (inactivated	Extract plus toxin 1:25000	Extract plus toxin 1:50000	Extract plus toxin 1:75000	Extract plus toxin 1:100000
Shellfish in tomato sauce	1,56	1,64	1,76	1,82	0,62	1,06	1,5	1,56
Gray mullet in oil	1,52	1,32	1,78	1,62	0,56	0,6	0,66	1,25
Pike perch (in tomato sauce)	1,74	1,64	1,94	1,76	0,58	0,9	1,02	1,28
Sprat in marinade	1,54	1,58	1,96	1,68	0,82	1,1	0,38	1,62
Goby in tomato sauce	1,46	1,24	1,78	1,8	0,62	0,88	0,82	1,0
Summer squash preserve	1,36	1,4	1,82	1,68	0,74	0,93	1,34	1,48
Eggplant preserve	1,62	1,72	1,83	1,73	0,58	0,76	0,92	1,28
Stuffed eggplants	1,64	2,14	2,4	1,98	0,52	0,74	0,86	0,84
Stewed beef	1,82	2,04	1,9	2,32	0,46	0,44	0,44	0,7
Stewed pork	1,46	1,88	1,74	2,26	0,3	0,56	0,64	0,96

Phagocytic index								
Extract plus toxin						Extract plus toxin (1:25) plus serum		
1:25	1:50	1:75	1:100	1:150	1:200	Immuno		Normal equine.
						Type A.	Type B.	
0,03	0,4	0,52	0,8	0,98	1,28	0,9	2,4	1
0,12	0,2	0,6	0,84	1	1,3	0,7	2,28	0,8
0,18	0,32	0,4	0,76	1,12	1,4	0,64	1,84	2,58
0,3	0,48	0,32	0,8	1,2	1,58	0,74	1,72	0,62
0,4	0,24	0,4	0,72	1,34	1,6	0,74	1,8	0,74
0,22	0,18	0,56	0,98	1,20	1,42	0,92	1,9	0,6
0,08	0,2	0,28	0,9	1,14	1,48	0,7	2,4	0,5
0,18	0,36	0,44	1,02	1,26	1,56	0,32	2,3	0,42
0,26	0,4	0,6	1	1,36	1,6	0,5	2,22	0,7
0,28	0,8	0,36	1	1,32	1,04	0,4	2,3	0,5
0,42	0,7	0,5	0,98	1,3	1,82	0,3	2,02	0,66
0,32	0,64	0,9	1,12	1,76	1,28	0,28	1,98	0,7
0,1	0,3	0,52	0,96	1,2	1,58	0,4	2	0,7
0,42	0,54	0,5	0,84	1,32	1,9	0,62	1,98	0,28
0,34	0,7	0,82	1,14	1,16	1,76	0,98	2,14	0,3

			Biological			
Extract plus toxin 1:25000 plus anti-serum A	Fxtract plus toxin 1:25000 plus anti-serum B.	Extract plus toxin 1:25000 plus normal equine serum	1 milliliter of preserved (canned) food	1 milliliter of inactivated canned food	1 milliliter of extract with toxin 1:25000 (inactivated	1 milliliter of extract plus toxin 1:25000
1,68	0,82	0,72	Alive	Alive	Alive	Died
1,4	0,86	0,74	"	"	"	"
1,82	0,72	0,84	"	"	"	"
1,8	0,94	0,88	"	"	"	"
1,72	0,84	0,7	"	"	"	"
1,48	0,9	0,96	"	"	"	"
1,74	0,98	0,78	"	"	"	"
2,36	0,76	0,56	"	"	"	"
1,52	0,74	0,5	"	"	"	"
1,78	0,5	0,68	"	"	"	"

Test					
1 milliliter of extract plus toxin 1:50000	1 milliliter of extract plus toxin 1:75000	1 milliliter of extract plus toxin 1:125000	0.7 milliliter of extract plus toxin plus 0.3 milliliter of A antiserum	0.7 milliliter of extract plus toxin plus 0.3 milliliter of B antiserum	0.7 milliliter of extract plus toxin, plus 0.3 milliliter of normal equine serum
Died	Alive	Alive	Alive	Died	Died
"	"	"	"	"	"
Ill, but alive	"	"	"	"	"
Died	"	"	"	"	"
Ill, but alive	Died	"	"	"	"
Died	Alive	"	"	"	"
Died	Died	"	"	"	"
Alive	Alive	"	"	"	"
"	"	"	"	"	"
"	"	"	"	"	"

T A B L E 2.

Name of the Product	Physiological solution	Native extract	Inactivated extract	Extract plus toxin 1:50 both inactivated
Stewed pork	1,78	1,3	2,3	1,92
Beef	1,82	2	2,42	1,83
Stuffed peppers	1,64	1,84	1,98	1,98
Summer squash preserves	1,82	1,68	1,88	1,8
Eggplant preserves	1,7	1,74	1,92	1,94
Codfish in oil	1,58	1,6	2	2,12
Cracow sausage	1,62	2,04	2,4	1,98
Poltava sausage	1,78	1,96	2,08	2,14
Tea sausage	1,93	2	2,18	2,06
Herring in marinade	1,7	1,56	1,96	2
" " "	1,84	1,72	1,88	1,94
" " "	1,84	1,8	1,92	1,8
Ham	1,94	1,88	1,98	2,02
"	1,88	1,92	2,02	2,32
"	1,72	2	2,08	2,26